

# Flavopiridol Protects Against Fungal Keratitis by Alleviating Inflammation Through the Promotion of Autophagy

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## Research Article

**Keywords:** flavopiridol, fungal keratitis, inflammation, autophagy, *Aspergillus fumigatus*

**Posted Date:** January 3rd, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1204241/v1>

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# Abstract

**Background:** Fungal keratitis is a serious infectious keratopathy related to fungal virulence and excessive inflammatory responses. Autophagy exhibits a potent ability to resolve inflammation during fungal infection. This study aimed to investigate the protective function of flavopiridol in fungal keratitis and explore its effects on autophagy.

**Methods:** A mouse model of fungal keratitis was established and then treated with 5  $\mu$ M flavopiridol. RAW 264.7 cells were treated with 200 nM flavopiridol before fungal stimulation. The severity of corneal diseases was evaluated by slit-lamp microscopy. The expression levels of cytokines were detected by RT-PCR and ELISA. The protein levels of LC3, Beclin-1 and Atg7 were determined by western blot and immunofluorescence. A Cell Counting Kit-8 assay was used to test cell viability. Autolysosomes were detected by transmission electron microscopy (TEM). An inhibitor of autophagy, 3-methyladenine (3-MA), was used to pretreat RAW 264.7 cells. Phagocytosis of RAW 264.7 cells was evaluated by counting colony forming units. *A. fumigatus* was incubated with flavopiridol, and the hyphae were stained with calcofluor white. Absorbance assay, crystal violet staining and adherence assay were used to detect the antifungal activity of flavopiridol.

**Results:** Flavopiridol treatment notably reduced corneal opacity and the clinical scores of infected corneas. Compared with DMSO treatment, flavopiridol treatment greatly downregulated IL-1 $\beta$ , IL-6 and TNF- $\alpha$  expression in infected corneas. In RAW 264.7 cells, flavopiridol treatment inhibited IL-1 $\beta$ , IL-6 and TNF- $\alpha$  expression but promoted IL-10 expression. TEM images showed that more autolysosomes were presented in infected corneas and RAW 264.7 cells after flavopiridol treatment than after DMSO treatment. Flavopiridol treatment notably upregulated the protein expression of LC3, Beclin-1 and Atg7 in infected corneas as well as in RAW 264.7 cells. 3-MA pretreatment counteracted the cytokine regulation induced by flavopiridol. Moreover, flavopiridol promoted the phagocytosis of RAW 264.7 cells. Flavopiridol also exhibited antifungal activity by restricting fungal growth and limiting fungal biofilm formation and conidial adhesion.

**Conclusions:** Flavopiridol significantly alleviated the inflammation of fungal keratitis by activating autophagy. In addition, flavopiridol promoted the phagocytosis of RAW 264.7 cells and exhibited antifungal function, indicating the potential therapeutic role of flavopiridol in fungal keratitis.

## Introduction

Fungal keratitis is a serious infectious keratopathy with a high incidence of vision loss, which is mainly caused by *Fusarium* and *Aspergillus* (Huang et al. 2016, Mahmoudi et al. 2018). Corneal trauma after agricultural injury and contact lens usage have been recognized as the predominant risk factors (Mahmoudi et al. 2018). The severity of fungal keratitis is related to fungal virulence and excessive inflammatory responses (Niu et al. 2019). However, due to the insufficiency and severe side effects of

antifungal drugs, fungal keratitis has a poor prognosis. Thus, it is urgent to explore the novel approaches to treat fungal keratitis.

Flavopiridol is a synthetic flavone that has been postulated to be an inhibitor of cyclin-dependent kinases (CDKs) (Schmerwitz et al. 2011). In addition to antitumor activities, CDK inhibitors (CDKi) show prominent anti-inflammatory effects (Leitch et al. 2009, Rossi et al. 2006). Among the CDKs, flavopiridol has a preference for suppression of CDK9 (Krystof et al. 2012). CDK9 regulates inflammation by interacting with p65 and inhibiting the IL-6/STAT3 signaling pathway (Hou et al. 2007, Hu et al. 2018). Currently, CDK9 inhibitors are considered potential treatments for inflammatory diseases (Hou et al. 2007). *In vivo*, CDKi, such as flavopiridol and R-roscovitine, exert anti-inflammatory and pro-resolution functions in pleurisy, hepatitis and pneumonia (Schmerwitz et al. 2011, Leitch et al. 2009, Rossi et al. 2006, Hoogendijk et al. 2012). *In vitro*, flavopiridol suppresses the interaction between leukocytes and endothelial cells in the model of murine hepatitis, which is a crucial step in the initiation of inflammation (Schmerwitz et al. 2011). In addition, CDKi exerts antiangiogenic functions in corneal diseases (Liebl et al. 2011). However, the anti-inflammatory function of flavopiridol has never been explored in fungal keratitis.

Although current studies have noted that flavopiridol inhibits the expression of inflammatory cytokines mainly through regulating the CDK9 and NF- $\kappa$ B signaling pathway, the specific anti-inflammatory mechanism of flavopiridol still remains unclear (Srikumar et al. 2016). Emilia Mahoney *et al.* demonstrated that flavopiridol could induce autophagy by increasing LC3 cytoplasmic accumulation and SQSTM1/p62 expression (Mahoney et al. 2013). Autophagy is a kind of degradation that removes long-lived proteins and organelles and is mediated by lysosomes. Autophagy is induced by various stimuli including starvation, stress, hypoxia and infection (Feng et al. 2014). Autophagy exhibits a potent ability to resolve inflammation, which is associated with the limitation and degradation of inflammasomes and cytokines (Galluzzi et al. 2017, Qian et al. 2017, Renga et al. 2018). These previous results implied that autophagy may become a novel target for flavopiridol to prevent inflammation. Macrophage autophagy can effectively degrade infectious microorganisms, thereby eliminating infection (Chai et al. 2016). A previous study has indicated that the promotion of autophagy could reduce the severity of fungal keratitis by regulating cytokines production (Li et al. 2020). Thus, we hypothesized that flavopiridol may exhibit a protective role in fungal keratitis by affecting autophagy.

Moreover, flavopiridol has been considered as an inhibitor of UDP-galactopyranose mutase (UGM) in *A. fumigatus*, suggesting its potential antifungal effect (Martín et al. 2017, Hostetter et al. 1994). UGM is a crucial enzyme for fungal cell wall biogenesis, which promotes the transformation from UDP-galactofuranose (Galf) to UDP-galactopyranose (Galp) (Latgé 2010). The fungal cell wall is involved in the interaction between pathogens and hosts, which determines the pathogenicity of fungal keratitis (Hostetter 1994). As an inhibitor of UGM, flavopiridol may inhibit the growth and virulence of fungi by restricting fungal cell wall formation. In addition, flavopiridol has been recognized as a candidate in infectious diseases (Ou et al. 2013, Nelson et al. 2001). Various studies have indicated that flavopiridol

inhibits viral transcription without cytotoxicity and alleviates virus-associated diseases, including adenoviral epidemic keratoconjunctivitis (Ou et al. 2013, Nelson et al. 2001).

Our study demonstrated that flavopiridol exerted anti-inflammatory activity by promoting autophagy in fungal keratitis. Additionally, flavopiridol could improve the phagocytosis ability of macrophages and restrict the growth of fungi. These data suggest that flavopiridol may exert therapeutic effects in fungal keratitis.

## Materials And Methods

### Mice corneal infection

8-week-old C57BL/6 female mice were provided by Jinan Pengyue corporation (Jinan, China). After anesthetization, the 2 mm-diameter central corneal epithelium of mice left eyes was scraped.  $10^8$  colony forming units (CFU) /mL *A. fumigatus* (5  $\mu$ L) was added on the surface of mouse corneas and then covered with a contact lens. All animal experiments abided by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Treatment of mice with flavopiridol

The left eyes of mice (n = 5/group) were treated with 5  $\mu$ L of flavopiridol (5  $\mu$ M; MCE, New Jersey, America) by subconjunctival injection at 1 day post infection (p.i.). Then, the left eyes were treated topically with 3  $\mu$ L of flavopiridol (5  $\mu$ M; MCE) twice a day from 2 day p.i. In control group, the left eyes of mice were treated with equivalent DMSO (Thermo Fisher Scientific, Waltham, MA, USA).

### Cell culture and fungal stimulation

The culture conditions for RAW 264.7 cells have been elaborated previously (Li et al. 2015). When the confluence of cells reached 80%, cells were transferred into serum-free DMEM and stimulated with *A. fumigatus* ( $5 \times 10^6$  CFU mL<sup>-1</sup>).

### Treatment of RAW 264.7 cells

Before fungal stimulation, RAW 264.7 cells were incubated with flavopiridol (200 nM; MCE) or DMSO for 2 hours. In 3-methyladenine (3-MA) group, RAW 264.7 cells were pre-treated with the inhibitor of autophagy, 3-MA (50  $\mu$ M; MCE), for 1 hour before flavopiridol treatment. The mRNA levels of cytokines including IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IL-10 were measured at 8 hours p.i. The protein levels of IL-6, TNF- $\alpha$  and IL-10 were measured at 24 hours p.i.

# Cell Counting Kit-8 assay

RAW 264.7 cells were incubated with flavopiridol (50, 100, 150 and 200 nM) or DMSO for 24 or 48 hours. Cell Counting Kit-8 assay (MCE) was used to detect the cell viability after incubation with flavopiridol.

## RT-PCR

Total RNA was extracted from mouse corneas and RAW 264.7 cells. The PCR method was described previously [25]. The primers used are exhibited in Table 1.

Table 1  
Primers used for RT-PCR

Gene	GenBank No.	Primer sequence (5'-3')
Mouse $\beta$ -actin	NM_007393.5	F: GATTACTGCTCTGGCTCCTAGC R: GACTCATCGTACTCCTGCTTGC
Mouse IL-1 $\beta$	NM_008361.4	F: CGCAGCAGCACATCAACAAGAGC R: TGTCCCTCATCCTGGAAGGTCCACG
Mouse IL-6	NM_001314054.1	F: TGATGGATGCTACCAAAGTGGG R: TGTGACTCCAGCTTATCTCTTGG
Mouse TNF- $\alpha$	NM_001278601.1	F: TTCTGTCTACTGAACTTCGGGGTGATCGGTCC R: GTATGAGATAGCAAATCGGCTGACGGTGTGGG
Mouse IL-10	NM_010548.2	F: TGCTAACCGACTCCTTAATGCA R: TTCTCACCCAGGGAATTCAAA

## ELISA

Normal corneas and infected corneas were homogenized in PBST with the protease inhibitor (MCE) and then centrifuged. RAW 264.7 cells supernatants were collected and centrifuged. Each sample was diluted to the appropriate concentration. The protein expressions of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IL-10 were detected by ELISA kits (BioLegend, CA, USA).

## Transmission electron microscopy

RAW 264.7 cells were treated with DMSO or flavopiridol for 2 hours, and then stimulated by fungi for 8 hours. Then cells were scraped off and placed in a microcentrifuge tube. After centrifuged at the speed of 3000 rpm for 10 minutes, cell precipitate was obtained and fixed in 2.5% glutaraldehyde. Mouse corneas

were removed and fixed in 2.5% glutaraldehyde. The preparation steps of samples before transmission electron microscopy (TEM) observation were described in the previous study (Liu et al. 2019).

## Immunofluorescence staining

RAW 264.7 cells were pretreated with 200 nM flavopiridol or DMSO for 2 hours, and then incubated with *A. fumigatus* for 8 hours. Cells were fixed on the poly-L-lysine-coated slips by 4% paraformaldehyde for 15 minutes. The immunofluorescence protocol was elaborated previously (Zhou et al. 2012). The anti-LC3B antibody (1:1000; Abcam, Cambridge, UK), anti-Beclin-1 antibody (1:200; Abcam) and anti-Atg-7 antibody (1:300; Abcam) were used as primary antibodies. The FITC-conjugated goat anti-rabbit antibody was used as the secondary antibody (1:200; Abcam).

## Western blot

Proteins were extracted from mouse corneas and cells by using RIPA lysis and extraction buffer (Thermo Fisher Scientific), 1 mmol/L phenylmethanesulfonyl fluoride (Solarbio, Beijing, China) and 1 mmol/L phosphatase inhibitor (MCE). The concentration of extracted protein was measured by the BCA protein quantification kit (Solarbio). After protein denaturation, electrophoresis and electrophoretic transfer, proteins were moved onto PVDF membranes (Jiang et al. 2020). The membranes were blocked by blocking agent (Thermo Fisher Scientific), and then incubated with primary antibodies and secondary antibodies. The primary antibodies used include  $\beta$ -actin antibody (1:1000; CST, USA),  $\beta$ -tubulin antibody (1:1000, CST), LC3B antibody (1:2000; Abcam), Beclin-1 antibody (1:2000; Abcam) and Atg-7 antibody (1:10000; Abcam).

## Phagocytosis

RAW 264.7 cells (100  $\mu$ L,  $1 \times 10^6$  cells/mL) were incubated with 200 nM flavopiridol or DMSO for 2 hours. Cells were centrifuged and then resuspended in 100  $\mu$ L DMEM. Next, cells were treated with equivalent numbers of conidia at 37 °C. At 0 min and 120 min, 50  $\mu$ L of the suspension described above was added to 150  $\mu$ L of HBSS solution. After centrifugation (110g/min, 4 min), 100  $\mu$ L supernatant was tiled on Sabouraud medium plate and the number of fungal colonies was counted. Phagocytosis was measured by calculating the reduction of ectocytic conidia left in the supernatant. The formula is as follows: phagocytic index P (120min) =  $(1 - N_{120}/N_0) \times 100\%$ .  $N_0$  and  $N_{120}$  represented the number of ectocytic conidia at 0 min and 120 min respectively (Perkhofer et al. 2007, Novakowski et al. 2017).

## *A. fumigatus* growth analysis

*A. fumigatus* strain 3.0772 was provided by The China General Microbiological Culture Collection Center (China). *A. fumigatus* was incubated with DMSO and flavopiridol (200, 400 and 800 nM; MCE) in 96-well plate for 1, 2, 3, 4 and 5 days. The absorbance of fungi was measured at 540 nm. Fungi was also stained with Calcofluor white (Sigma, Santa Clara, CA, USA) for 10 minutes. The images of stained-hyphae were captured and the fluorescence intensity was measured.

## Fungal biofilm formation assay

The preparation of fungal biofilm formation has been described in previous publications (Wiederhold et al. 2018). The fungal biofilms were incubated with DMSO and flavopiridol (200, 400 and 800 nM) for 48 hours. Biofilms were fixed with methanol and stained by 0.1% crystal violet. After washed for three times, ethanol was used to release crystal violet bounded to biofilms. The absorbance was detected at 570 nm three times.

## Fungal adherence assay

HCECs ( $2 \times 10^4$ /mL) were treated with the mixture of conidia suspension (at an MOI of 10) and 200 nM flavopiridol or DMSO, and plated on chambered slides (Cameron et al. 1988). After incubation for 3 hours at 37°C, hematoxylin and eosin (HE) were used to stain the conidia and cells. The images of adherent conidia to HCECs were captured by microscopy (Thermo Fisher Scientific, 600×).

## Statistical analysis

Student's *t*-test was used to analyze differences between two groups. One-way ANOVA was used to evaluate differences among three or more groups. Differences were considered significant at  $P \leq 0.05$ . All data are shown as the mean  $\pm$  SEM.

## Results

### Flavopiridol ameliorates inflammation in fungal keratitis

Mouse corneas treated with 5  $\mu$ M flavopiridol or DMSO were photographed by the slit-lamp microscopy. The photos exhibited that flavopiridol treatment ameliorated corneal edema and opacity in infected corneas compared with DMSO treatment (Fig. 1a). Clinical scores were recorded by assessing the severity of fungal keratitis. The results indicated that flavopiridol significantly reduced the clinical scores of mouse corneas at 3 days p.i. (Fig. 1b;  $P < 0.001$ ). IL-1 $\beta$  (Fig. 1c;  $P < 0.01$ ), IL-6 (Fig. 1d;  $P < 0.05$ ) and TNF- $\alpha$  (Fig. 1e;  $P < 0.05$ ) mRNA levels were decreased in 5  $\mu$ M flavopiridol-treated corneas at 3 days p.i. ELISA was used to quantify the protein levels. ELISA results showed that flavopiridol treatment notably

decreased the protein levels of IL-1 $\beta$  (Fig. 1f;  $P < 0.001$ ), IL-6 (Fig. 1g;  $P < 0.001$ ) and TNF- $\alpha$  (Fig. 1h;  $P < 0.01$ ) in infected corneas at 3 days p.i.

## Flavopiridol regulated cytokine production in RAW 264.7 cells

RAW 264.7 cells were incubated with 50, 100, 150 and 200 nM flavopiridol for 24 and 48 hours, and the cell viability remained unchanged (Fig. 2a). Thus, 200 nM was chosen as the treatment concentration for the subsequent cellular experiments. Flavopiridol-treated cells showed lower mRNA levels of IL-1 $\beta$  (Fig. 2b), IL-6 (Fig. 2c) and TNF- $\alpha$  (Fig. 2e) but more IL-10 (Fig. 2g) than that in DMSO-treated RAW 264.7 cells at 8 hours p.i. ELISA results demonstrated that flavopiridol significantly downregulated IL-6 (Fig. 2d) and TNF- $\alpha$  (Fig. 2f) protein levels but upregulated the IL-10 protein level (Fig. 2h) in fungi-infected RAW 264.7 cells, compared with DMSO treatment.

## Flavopiridol induced autophagy in fungal keratitis

To detect the effect of flavopiridol on autophagy in corneal fungal infection, autolysosomes were observed by TEM. No autolysosomes were observed in images of normal mouse cornea (Fig. 3a) and normal mitochondria was observed at higher magnification (Fig. 3d). Few autolysosomes could be seen in infected cornea after DMSO treatment at 3 days p.i. (Fig. 3b). While, the cristae of mitochondria decreased or disappeared in infected cornea (Fig. 3e). As shown in Figs. 3c and 3f, more autolysosomes were formed and the number of mitochondria cristae increased in infected corneas after flavopiridol treatment.

To further explore the effects of flavopiridol on autophagy, the expression of LC3B  $\beta$ , LC3B  $\alpha$ , Beclin-1 and Atg-7 in infected corneas was detected. DMSO-treated corneas were set as the control group. Compared with control corneas, LC3B  $\beta$ , Beclin-1 and Atg-7 protein expression was increased in infected corneas. Flavopiridol treatment further elevated LC3B  $\beta$ , Beclin-1 and Atg-7 protein expression in infected corneas, compared with DMSO treatment (Figs. 3g-3l).

## Flavopiridol induced autophagy in RAW 264.7 cells

Autolysosomes in RAW 264.7 cells were detected by TEM. Fig. 4a displays the TEM image of a normal RAW 264.7 cell. After fungal stimulation for 8 hours, phagocytosed fungus and autolysosomes could be seen in the cytoplasm of RAW 264.7 cell (Fig. 4b). In the flavopiridol group, RAW 264.7 cells were incubated with 200 nM flavopiridol and then infected with *A. fumigatus* for 8 hours. Phagocytosed fungus (Fig. 4c) and an increased number of autolysosomes (Fig. 4d) were observed in RAW 264.7 cell in the flavopiridol group. To investigate the effects of flavopiridol on autophagy, the protein expression levels of LC3, Beclin-1 and Atg-7 were detected. LC3 expression in RAW 264.7 cells was detected by

immunofluorescence staining (Fig. 4e). DMSO-treated cells were set as the control group. The images showed that minimal LC3 was expressed in the control group. The immunofluorescence of LC3 was increased in infected RAW 264.7 cells after DMSO treatment and was mainly distributed in the cytoplasm. Compared with DMSO treatment, flavopiridol treatment further improved LC3 expression in infected RAW 264.7 cells. Next, Beclin-1 and Atg-7 protein expression levels in RAW 264.7 cells were measured. Western blot results showed that, fungal stimulation upregulated the protein levels of Beclin-1 (Figs. 4f, 4g;  $P \leq 0.05$ ) and Atg-7 (Figs. 4h, 4i;  $P \leq 0.001$ ) in RAW 264.7 cells. In addition, the protein levels of Beclin-1 (Figs. 4f, 4g;  $P \leq 0.05$ ) and Atg-7 (Figs. 4h, 4i;  $P \leq 0.05$ ) were both further elevated after flavopiridol treatment in infected cells. Immunostaining results demonstrated that both Beclin-1 (Fig. 4j) and Atg-7 (Fig. 4k) expression increased after *A. fumigatus* stimulation. Flavopiridol treatment further notably enhanced the fluorescence of Beclin-1 and Atg-7, compared with DMSO treatment.

## Autophagy inhibition restricted the anti-inflammatory activity of flavopiridol

To demonstrate the role of autophagy during the anti-inflammatory process of flavopiridol, 3-MA, an inhibitor of autophagy, was used to pretreat RAW 264.7 cells before flavopiridol treatment and *A. fumigatus* stimulation. The mRNA expression and protein levels of cytokines were examined by RT-PCR and ELISA. As shown in Figs. 8a and 8b, 3-MA treatment dampened the flavopiridol-induced downregulation of TNF- $\alpha$  at both the mRNA (Fig. 5a;  $P \leq 0.01$ ) and protein (Fig. 5b;  $P \leq 0.05$ ) levels in infected RAW 264.7 cells. In addition, compared with flavopiridol treatment, the mRNA (Fig. 5c;  $P \leq 0.01$ ) and protein (Fig. 5d;  $P \leq 0.001$ ) expression levels of IL-10 were reduced in infected RAW 264.7 cells in the 3-MA pretreatment group.

## Flavopiridol enhanced the phagocytosis of RAW 264.7 cells

To assess the effect of flavopiridol on the phagocytosis of RAW 264.7 cells, cells were treated with 200 nM flavopiridol and an equivalent number of conidia. The ectocytic conidia remained in the supernatant and the number of CFUs was counted (Fig. 6a). Phagocytosis was measured by the formula described in the methods. Compared with DMSO treatment, flavopiridol treatment notably increased the P (120 min) of RAW 264.7 cells (Fig. 6b;  $P \leq 0.01$ ).

## Flavopiridol suppresses the growth, biofilm formation, and adhesion ability of *A. fumigatus*

To test the antifungal effects of flavopiridol, *A. fumigatus* was incubated with 0, 200, 400 and 800 nM flavopiridol for 1, 2, 3, 4 and 5 days (Fig. 7a). After incubation with flavopiridol for 2 days, the absorbance of the medium and the fungal mass were measured. Compared with DMSO treatment, flavopiridol

significantly decreased the absorbance (Fig. 7b;  $P<0.05$ ;  $P<0.001$ ;  $P<0.001$ ). The hyphae were stained with calcofluor white, and images of stained hyphae were photographed. The images showed that fewer hyphae were present after 200, 400 and 800 nM flavopiridol treatment for 2 days compared to DMSO treatment (Fig. 7e). The fungal mass was quantified by assessing the fluorescence intensity of the stained hyphae. The fungal mass was reduced after flavopiridol treatment (Fig. 7c;  $P<0.001$ ;  $P<0.001$ ;  $P<0.001$ ). Flavopiridol restricted the biofilm formation of *A. fumigatus* at 200, 400 and 800 nM (Fig. 7d). In addition, HE staining was used to show the conidia that were adherent to HCECs. Because 200 nM flavopiridol exhibited no significant cytotoxic effect on HCECs, cells were treated with 200 nM flavopiridol. HE staining demonstrated that fewer conidia adhered to cells after flavopiridol treatment compared to DMSO treatment (Figs. 7f, 7g).

## Discussion

Fungal keratitis is a serious corneal disease caused by pathogenic fungi. Excessive inflammatory response resulted in the prolonged release of inflammatory factors, enzymes and toxic free radicals, causing corneal tissue damage (Gu et al. 2020). Pathogenic microorganisms such as fungi could induce autophagy process. Autophagy of macrophages is beneficial for cellular survival and resistance to infection. Thus, induction of autophagy has been considered as a potent method to alleviate inflammation. Flavopiridol is a synthetic flavonoid with numerous functions. Previous studies have demonstrated that flavopiridol could dramatically alleviate the severity of inflammatory diseases and promote autophagy activity (Schmerwitz et al. 2011, Haque et al. 2011, Haque et al. 2013, Hu et al. 2018). In addition, flavopiridol has been considered an inhibitor of UGM, which has antifungal potential (Martín et al. 2017). Thus, we hypothesized that flavopiridol might protect against fungal keratitis.

In our study, photographs taken by a slit-lamp camera demonstrated infected corneas with reduced edema and opacity following flavopiridol treatment. Additionally, the clinical scores were notably decreased after flavopiridol treatment. These results indicate that flavopiridol exhibits a protective role in fungal keratitis. Compared with DMSO treatment, flavopiridol treatment notably downregulated cytokines expression, including IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , in infected corneas. Our results are consistent with findings of prior studies showing that flavopiridol suppressed IL-6 and IL-1 $\beta$  production in postinjury cartilage explants and prevented injury after knee trauma (Hu et al. 2016). Flavopiridol has been verified to exert an anti-inflammatory function by reducing the induction and transactivation of cytokines (Hu et al. 2018). *In vitro*, we further tested the anti-inflammatory function of flavopiridol in RAW 264.7 cells. In prior studies, flavopiridol was demonstrated to inhibit LPS-induced cytokines production via a MyD88-dependent pathway in RAW 264.7 cells (Haque et al. 2011). Our results showed that flavopiridol significantly downregulated the levels of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  but enhanced the level of the anti-inflammatory cytokine IL-10 in *A. fumigatus*-stimulated RAW 264.7 cells. Based on these results, flavopiridol may represent a novel approach for alleviating inflammation in fungal keratitis.

Previous studies have highlighted the crucial role of autophagy in innate immunity during fungal infection (Li et al. 2020, Kanayama et al. 2015). During infection, autophagy helps to eliminate

intracellular pathogens and present antigens, and protects normal cells from being infected (Xian et al. 2016, Lu et al. 2017, Ferreira et al. 2013). At the beginning, phagophores are formed inside the cell. After phagocytizing the damaged organelles and denatured proteins, autophagosomes are formed. Then, autophagosomes bind with lysosomes to form autolysosomes, which help to degrade organelles, proteins and invading microorganisms (Galluzzi et al. 2019). In our study, TEM images exhibited no autolysosome in normal mice cornea or RAW 264.7 cells, while increased autolysosomes were present in infected corneas or RAW 264.7 cells. In addition, the cristae of mitochondria decreased or disappeared in infected corneas. Previous studies showed that flavopiridol induced autophagy by upregulating LC3B-II and downregulating p62 expression, which exerted a cytoprotective role (Wang et al. 2017, Okada et al. 2017, Jeong et al. 2018). Our results showed that flavopiridol treatment further increased autolysosomes in infected mouse corneas or RAW 264.7 cells, compared with DMSO treatment. The number of mitochondria cristae were back to normal after flavopiridol treatment. During autophagy, LC3-I is modified and processed by a ubiquitin-like system including Atg7 and Atg3 to form LC3-II, which locates on the membrane of autophagosome. The expression of LC3 has been considered as a crucial criterion for detecting autophagy. In addition, Beclin-1 and Atg7 are crucial for autophagic responses (Liu et al. 2019). Thus, the expressions of LC3, Beclin-1 and Atg7 were examined in fungal keratitis. Compared with uninfected corneas and RAW 264.7 cells, the protein levels of LC3B II, Beclin-1 and Atg-7 were increased after fungal stimulation. Our results indicated that autophagy is promoted in fungal keratitis, which is consistent to the previous study (Li et al. 2020). We found that flavopiridol treatment could further elevate protein levels of LC3B II, Beclin-1 and Atg-7 in infected corneas and RAW 264.7 cells, implying that flavopiridol upregulated autophagy in fungal keratitis. Autophagy has been considered as a significant player in regulating inflammation during fungal infection. In our study, 3-MA, an inhibitor of autophagy, was used to pretreat RAW 264.7 cells before flavopiridol treatment. 3-MA could specifically block the fusion of autophagosomes and lysosomes, inhibiting the formation of autolysosomes. Our study showed that 3-MA pretreatment significantly counteracted the downregulation of TNF- $\alpha$  and upregulation of IL-10 induced by flavopiridol in infected RAW 264.7 cells. Thus, flavopiridol exerted anti-inflammatory effects by inducing autophagy in fungal keratitis. In addition to autophagy, macrophages also exhibit phagocytosis ability, which can directly phagocytose fungi and participate in the innate immune process of fungal keratitis. Our results indicated that flavopiridol treatment increased macrophage phagocytosis, contributing to the elimination of fungi.

In addition, flavopiridol exhibits potential antifungal effects. Galactomannan, the production of which is initiated by UGM, is a ubiquitous component in fungal cell walls. The absence of UGM leads to thinner fungal cell wall and reduced fungal virulence (Martín et al. 2017, Schmalhorst et al. 2008). In our study, *A. fumigatus* was cultivated with different concentrations of flavopiridol for 1, 2, 3, 4 and 5 days. The absorbance of the medium was reduced after flavopiridol treatment. Fungal mass results and images of immunostained fungi indicated that, flavopiridol treatment reduced the amount of *A. fumigatus* in a concentration-dependent manner. Our results further demonstrated that flavopiridol treatment effectively decreased fungal biofilm formation and restricted the adhesion of conidia to HCECs. Biofilm formation and adhesion are crucial for fungi to initiate infection and resist the immune system (Taylor et al. 2014,

Beauvais et al. 2015). These results provide evidence that flavopiridol exerts antifungal effects in fungal keratitis.

In conclusion, flavopiridol protects against fungi-induced corneal damage by suppressing excessive inflammatory responses and limiting fungal growth. Our study demonstrated that flavopiridol upregulated autophagy activity and improved the phagocytosis ability of macrophages, contributing to the anti-inflammatory function and protective role of flavopiridol in fungal keratitis.

## Abbreviations

*A. fumigatus*: *Aspergillus fumigatus*; CDKs: cyclin-dependent kinases; CDKi: CDK inhibitors; 3-MA: 3-methyladenine; TEM: transmission electron microscopy; HE: hematoxylin and eosin; CFU: colony forming units.

## Declarations

## Acknowledgements

Not applicable.

## Authors' contributions

GZ and JL designed the study. LG carried out experiments and wrote the manuscript. HL and YN contributed to the data analysis. CL, XP and HZ were responsible for revising the paper.

## Funding

This work was financially supported by the National Natural Science Foundation of China (Nos. 81870632 and 81800800), the Taishan Scholars Program (Nos. ts201511108 and tsqn201812151).

## Availability of data and materials

The datasets used during this study are available from the corresponding author on reasonable request.

## Ethical approval and consent to participate

All animal experiments abided by the Animal Care and Use Committee of the Affiliated Hospital of Qingdao University and the experiments were performed in strict adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

# Consent for publication

Not applicable.

# Competing interests

The authors declared no conflict of interest.

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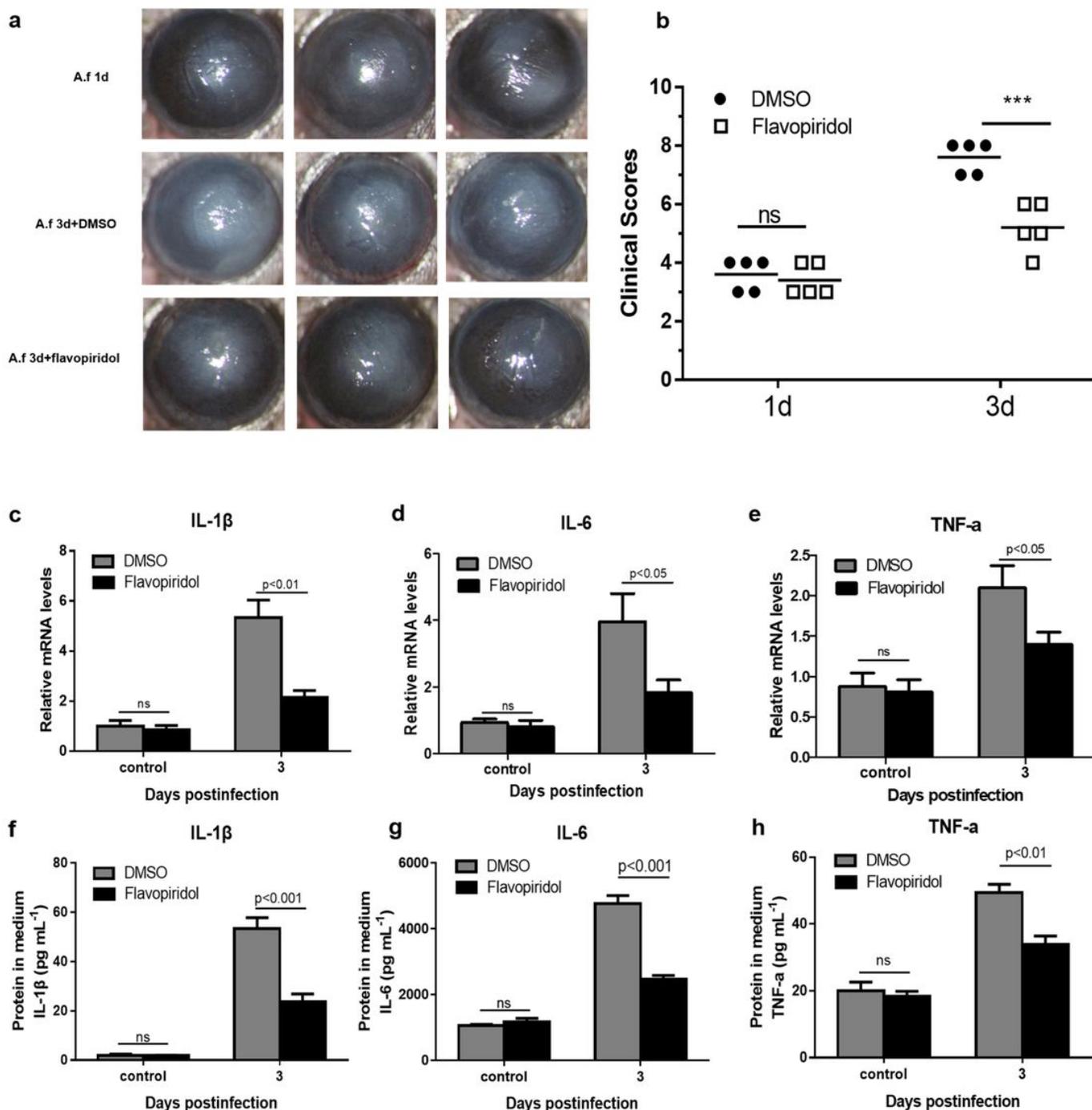
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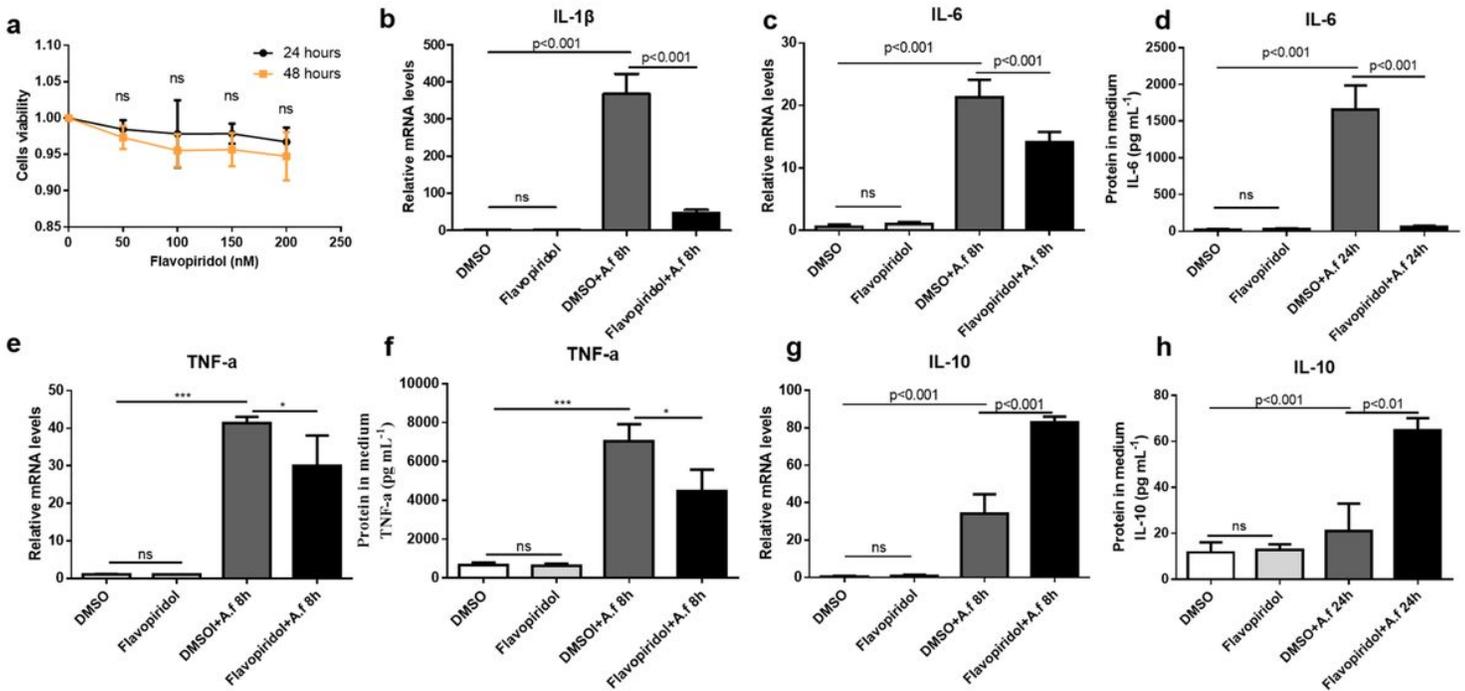
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## Figures



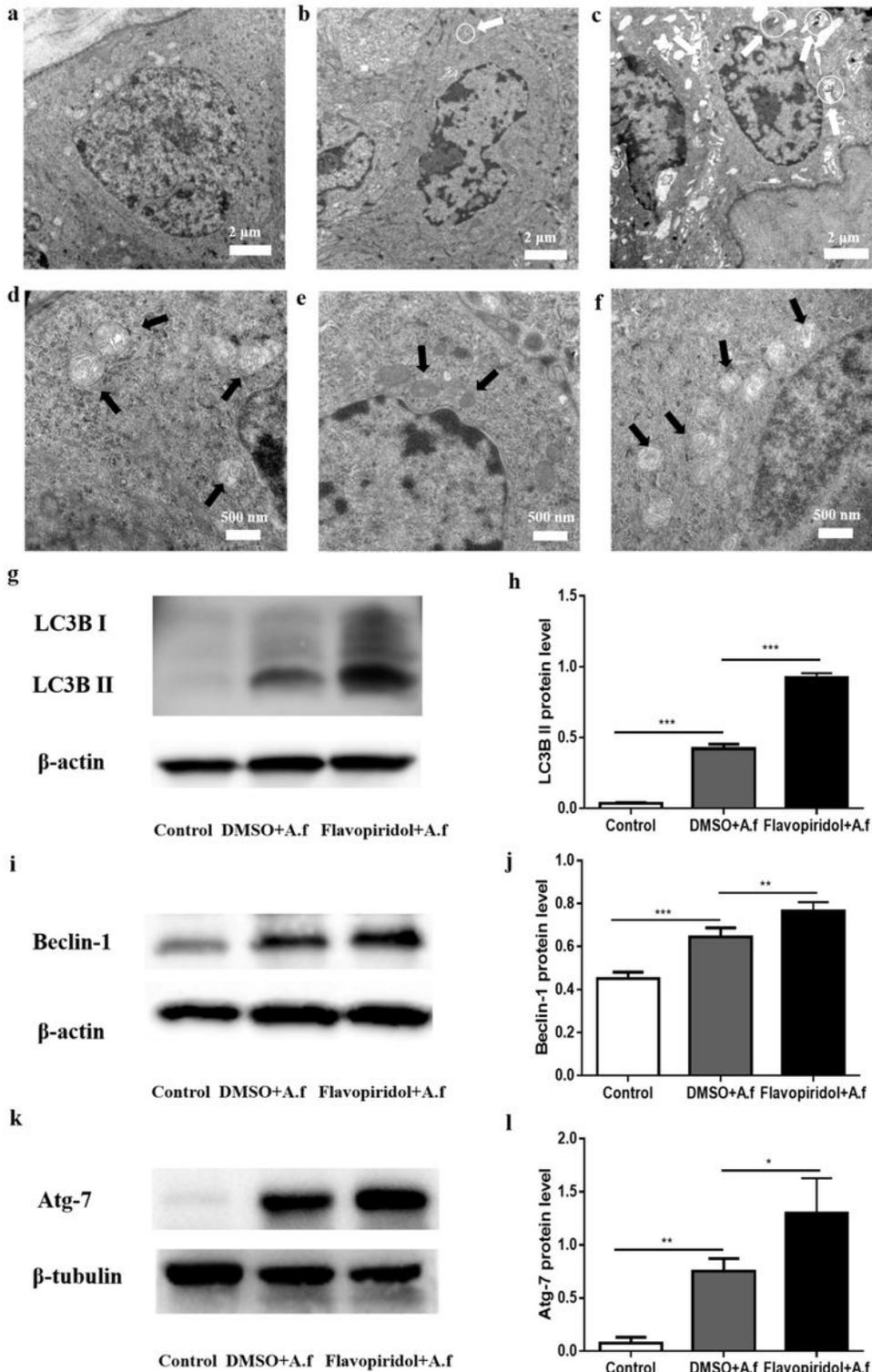
**Figure 1**

Flavopiridol treatment alleviates inflammation of fungal keratitis. The photographs (n = 5) taken by slit-lamp camera showed that flavopiridol treatment reduced the corneal edema and ulceration at 3 days p.i., compared with DMSO treatment. Magnification: × 25 (a). Flavopiridol-treated infected corneas (n = 5) showed less clinical scores than that of DMSO-treated infected corneas at 3 days p.i. (b). Flavopiridol treatment markedly reduced the mRNA levels of IL-1 $\beta$  (c), IL-6 (d) and TNF-a (e) in infected corneas (n = 5) at 3 days p.i. ELISA results demonstrated that the protein levels of IL-1 $\beta$  (f), IL-6 (g) and TNF-a (h) in corneas at 3 days p.i. were also downregulated in flavopiridol group.



**Figure 2**

Flavopiridol treatment downregulates cytokine levels in RAW 264.7 cells. The cells viability remained unchanged with 50, 100, 150 and 200 nM flavopiridol treatment for 24 and 48 hours (a). The mRNA levels of IL-1 $\beta$  (b), IL-6 (c) and TNF-a (e) were reduced after flavopiridol treatment in *A. fumigatus* stimulated RAW 264.7 cells for 8 hours, compared with DMSO treatment. However, the mRNA level of IL-10 (g) was upregulated after flavopiridol treatment compared with DMSO treatment. ELISA results showed that flavopiridol inhibited protein expression of IL-6 (d) and TNF-a (f), whereas promoted protein production of IL-10 (h) in infected cells for 24 hours.



**Figure 3**

Flavopiridol treatment induced autophagy in infected corneas. TEM image of normal mouse cornea (**a**). Autolysosome was shown in *A. fumigatus* infected cornea. The white arrow indicates the autolysosome (**b**). Increased autolysosomes were observed in flavopiridol-treated infected cornea. The white arrows denote autolysosomes (**c**). Normal mitochondria could be observed in the cytoplasm of normal corneal epithelial cell at higher magnification of Fig. 3a. The black arrows denote the normal mitochondria (**d**).

The cristae of mitochondria decreased or disappeared in *A. fumigatus* infected cornea. The black arrows denote the mitochondria (e). Mitochondria cristae were dense in flavopiridol-treated infected corneal epithelium. The black arrows denote the mitochondria (f). Flavopiridol induced LC3B  $\square$ , LC3B  $\square$  (g, h), Beclin-1 (i, j) and Atg-7 (k, l) protein expression in infected mouse corneas.

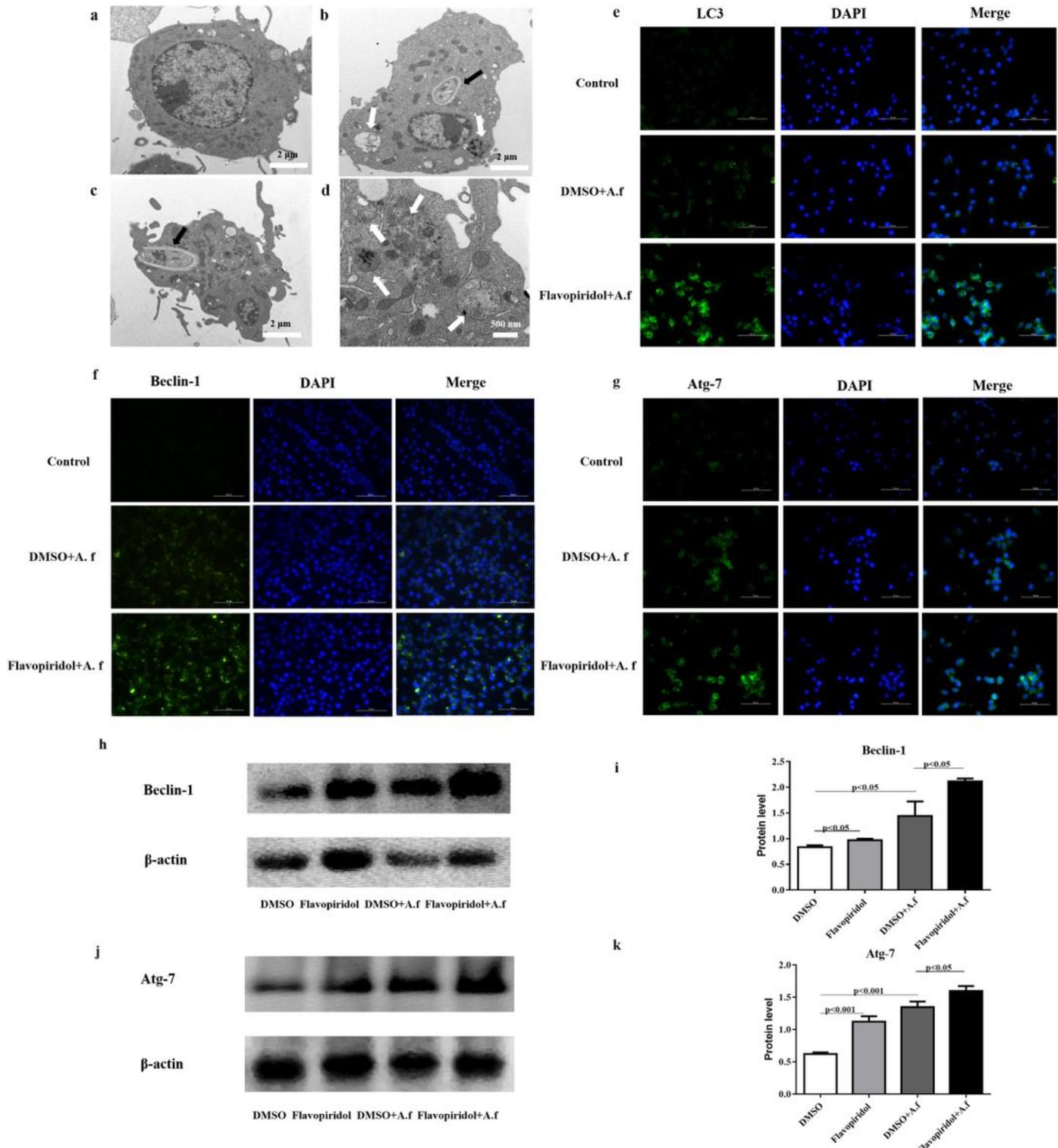
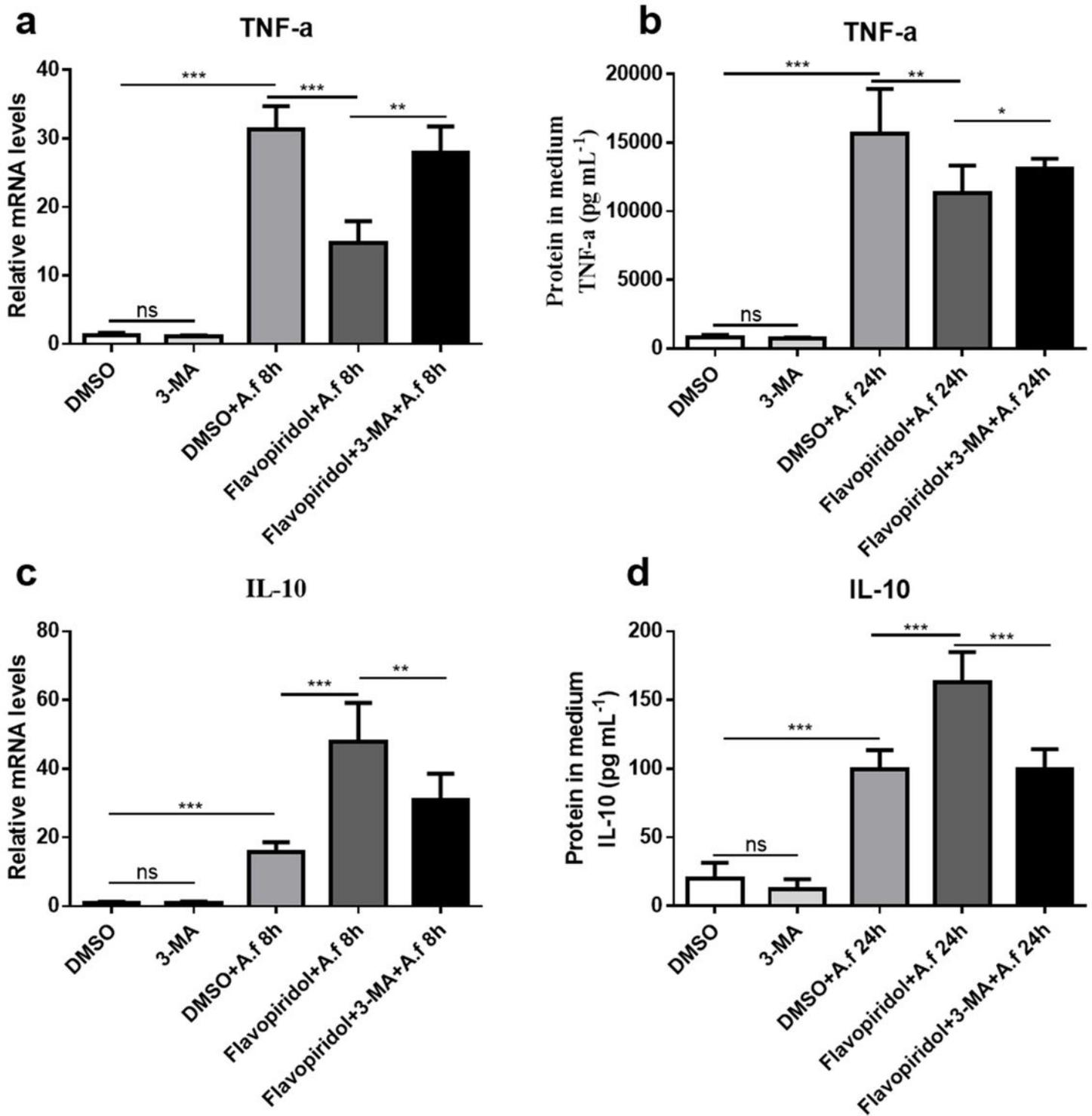


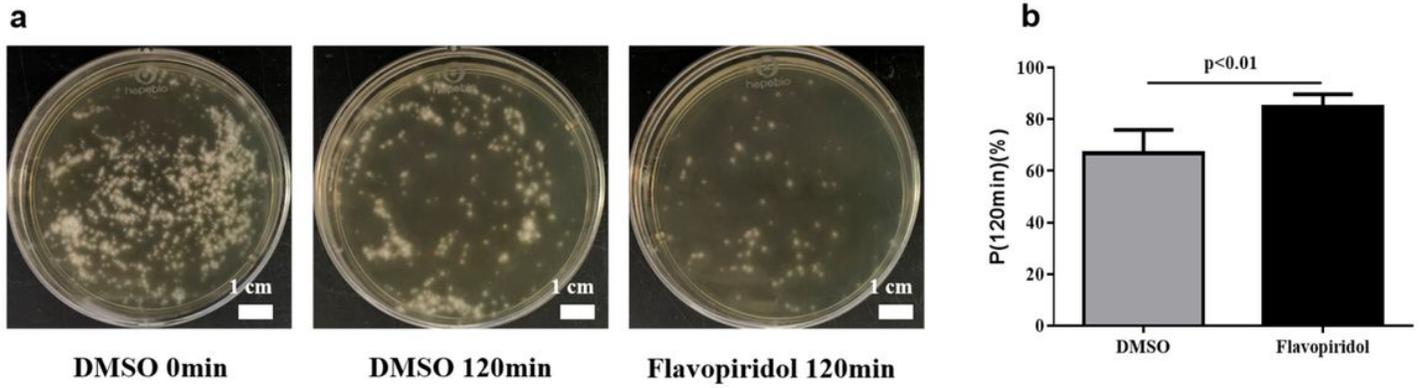
Figure 4

Flavopiridol treatment induced autophagy in infected RAW 264.7 cells. TEM image of normal RAW 264.7 cell (**a**). TEM image of *A. fumigatus* infected RAW 264.7 cell. The black arrow indicates the fungus swallowed by RAW 264.7 cell and the white arrows indicate autolysosomes (**b**). TEM image of flavopiridol pre-treated infected RAW 264.7 cell. The black arrow indicates the fungus swallowed by RAW 264.7 cell (**c**). The autolysosomes were shown in flavopiridol pre-treated infected RAW 264.7 cell at higher magnification of Fig. 4c. The white arrows denote the autolysosomes (**d**). The images of immunofluorescence staining showed that flavopiridol improved LC3 (**e**), Beclin-1 (**f**) and Atg-7 (**g**) expression in infected RAW 264.7 cells. Magnification: × 400. Flavopiridol increased Beclin-1 (**h, i**) and Atg-7 (**j, k**) protein expression in infected RAW 264.7 cells at 8 hours p.i.



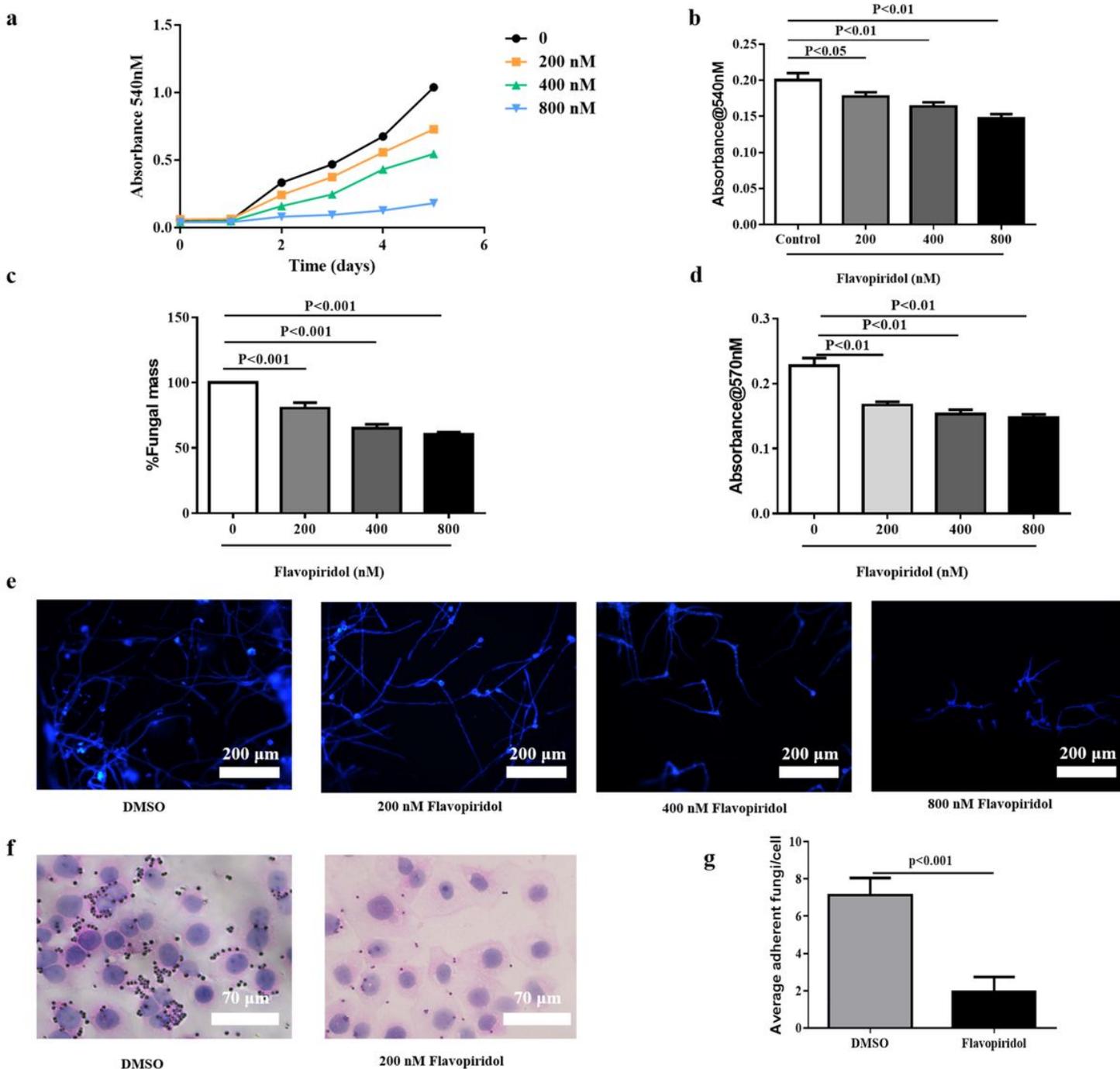
**Figure 5**

Effects of autophagy on the anti-inflammatory function of flavopiridol in RAW 264.7 cells. 3-MA, an inhibitor of autophagy, was used to pretreat RAW 264.7 cells. Compared with flavopiridol treatment, 3-MA pretreatment before flavopiridol treatment significantly increased the mRNA (a) and protein (b) levels of TNF-a in infected RAW 264.7 cells. Compared with flavopiridol treatment, 3-MA pretreatment notably reduced the mRNA (c) and protein (d) levels of IL-10 in infected RAW 264.7 cells.



**Figure 6**

Effects of flavopiridol on the phagocytosis of RAW 264.7 cells. The CFU of the ectocytic live *A. fumigatus* conidia (**a**). P (120min) of RAW 264.7 cells after DMSO treatment or flavopiridol treatment. Compared with DMSO treatment, flavopiridol treatment significantly increased the phagocytic index of RAW 264.7 cells (**b**).



**Figure 7**

Effects of flavopiridol on fungal growth, biofilm formation and adherence. Treatment with different concentrations of flavopiridol for 1, 2, 3, 4 and 5 days decreased the absorbance of the medium (a). 200, 400 and 800 nM flavopiridol treatment for 2 days notably reduced the absorbance of *A. fumigatus* (b). The fungal mass was also decreased after 200, 400 and 800 nM flavopiridol treatment for 2 days (c). Flavopiridol significantly inhibited fungal biofilm formation at 200, 400 and 800 nM, as determined by analyzing the amount of crystal violet from biofilms (d). Few hyphae were present after high concentrations of flavopiridol treatment, as shown in images of stained hyphae. Magnification:  $\times 100$ .

(e). HE staining showed fewer conidia adherent to HCECs after flavopiridol treatment compared with DMSO treatment. Magnification: × 600. (f). The amount of adherent conidia was also quantified (g).